AFIRM NERVE REGENERATION PROJECT 1: GROWTH FACTOR RELEASING MICROSPHERES AND THE EFFECT ON PERIPHERAL NERVE REGENERATION

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1. INTRODUCTION

The current gold standard for repair of a nerve injury is the interposition of an autologous nerve graft. This has several disadvantages, such as donor site morbidity, limited availability, size mismatch and painful neuroma formation. In addition, functional recovery rarely returns to pre-injury level. Therefore, the development and optimization of nerve conduits is an increasingly important focus in the field of peripheral nerve regeneration. One promising approach that may enhance nerve regeneration is the long term delivery of growth factors. However, accurate delivery and concentration maintenance of growth factors within the nerve conduit still pose problems. We therefore studied the long term release of nerve growth factor (NGF) using a microsphere delivery system. First, we evaluated the release of NGF from five different types of polymer microspheres both in vitro and in vivo. Next, we assessed the effect of both NGF and glial cell-line derived neurotrophic factor (GDNF) releasing microspheres on functional regeneration using polymer nerve conduits.

2. METHODS

1. Microsphere Selection

Polymer microspheres were fabricated using the double emulsion, solvent evaporation technique. Five types of poly-lactic-co-glycolic-acid (PLGA) were tested: 50:50 1A (inherent viscosity 0.1dL/g), 50:50 4A (inherent viscosity 0.4dL/g), 50:50 7A (inherent viscosity 0.7dL/g), 75:25 (inherent viscosity 0.7dL/g) and 85:15 (inherent viscosity 0.7dL/g). NGF was radiolabeled with iodine 125 and incorporated into the microspheres. Sphere size and degradation profiles were assessed over time using scanning electron microscopy. Encapsulation efficiency and in vitro release profiles were assessed over a period of 8 weeks by γ-counting. Biological activity of released NGF was verified using a E15 dorsal root ganglion bioassay. In vivo release profiles of microspheres were evaluated by subcutaneous implantation of microspheres in nerve conduits and measurements of radioactivity for a duration of 8 weeks.

2. Functional Regeneration

PLGA nerve conduits were used to bridge a 10 mm gap in a rat sciatic nerve injury model (Fig. 1)

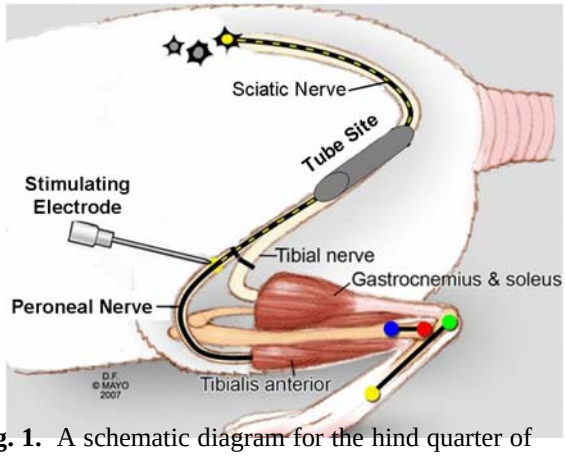

Fig. 1. A schematic diagram for the hind quarter of a rat showing the location of the nerve tube and motion analyses points.

Conduits were fabricated by an injection molding technique resulting in conduits with an inner diameter of 1.6mm. Conduits were implanted at mid-thigh level. Nerve conduits were loaded with either GDNF, NGF, GDNF-microspheres or NGF-microspheres at 3 different concentrations. An autograft and a conduit filled with saline were used for comparison. Electrophysiological (CMAP) measurements of tibial and peroneal innervated muscles as well as motor testing (2D video motion analysis) were carried out at baseline, directly postoperatively and at regular intervals after surgery. For motor testing, ankle angle

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measurements were made during rat walking cycles (initial contact, toe off and midswing).
At 16 weeks, nerves were sectioned distal to the implantation site and the retrograde tracer fast blue was applied for 30 minutes. Five days later, appropriate spinal cord segments and dorsal root ganglion neurons were harvested and processed for cell counting. Also, sciatic nerves were analyzed for nerve morphometry.

3. RESULTS

1. Microsphere Selection

Encapsulation efficiency was highest (74.6%) for the microsphere type with a PLGA ratio of 50:50, with an inherent viscosity of 0.1dL/g. Encapsulation efficiency was most dependent on the inherent viscosity of the polymer (35.6% - 74.6%). Differences in ratio of lactic to glycolic acid did not change encapsulation efficiency substantially. It varied from 26.9% (75:25 7A) to 35.6%. (50:50 7A). (Figure 2a)

Microsphere size distribution was similar for polymer types 50:50 4A, 50:50 7A, 75:25 and 85:15, (figure 2b) showing 39.6% - 48.5% of spheres to be 9µm or smaller. Polymer type 50:50 1A differed by showing a peak of 35.2% of its total in the 10-19µm range and having only 22.4% smaller than 9µm. Degradation profiles showed complete degradation after three weeks for polymer types 50:50 1A and 50:50 4A. Spheres of ratios 75:25 and 85:15 kept their integrity for over five weeks.

NGF was gradually released from all microspheres at a rate of 0.6-1% of total per day in vitro (figure 2c). A small (<5%) burst release profile from loosely bound NGF, was seen in all microspheres during the first 24h. A second release peak occurred from all polymers at 3 weeks due to mass degradation of PLGA. DRG bioassays confirmed biological activity of the released NGF for a duration of 23 days (Figure 2d).

In vivo, microspheres showed a faster release than in vitro. Depending on the type of polymer, release rates varied between 1% (85:15 7A) and 2.5% (50:50 1A) of total per day (Figure 3).

Based on degradation and release profiles, microspheres fabricated from polymer type 50:50 4A seemed best suited for use in an in vivo rat nerve regeneration model.

2. Functional Regeneration

All nerve tubes showed a regenerative nerve cable at endpoint and no sutures were pulled out from nerve conduits during the study (Figure 4).

CMAP measurements returned after 8 weeks in the autologous group and at 10 weeks in all other groups (Figure 5). Tibial CMAP amplitude continued to increase until endpoint, with saline, saline NGF, GDNF, NGF microsphere and GDNF microsphere groups varying between 2.6mV – 3.8mV and showing equal or higher values than the autologous group (2.5mV) (Figure 5 b and c).

Motor testing showed a postoperative decrease in ankle angle in all groups for initial contact and toe off, followed by a continuous increase after week 8. Ankle angle at midswing remained steady after surgery and started to increase from week 8 until endpoint. No differences were found at endpoint between all evaluated groups (figure 6).

Motoneuron counting, along with sciatic nerve morphometric measurements are being evaluated.

3. Conclusion

PLGA microspheres are able to release active growth factor for a duration of 23 days. Based on degradation and release profiles, microspheres fabricated from PLGA 50:50 4A were found best suited for use in peripheral nerve conduits.

In vivo, PLGA conduits provide excellent scaffolds for peripheral nerve repair. These conduits can be used to optimize delivery conditions for growth factors such as NGF and GDNF. Currently we achieved results comparable to autologous grafting, based on electrophysiological and functional tests. Further modification of conduits may further enhance nerve regeneration

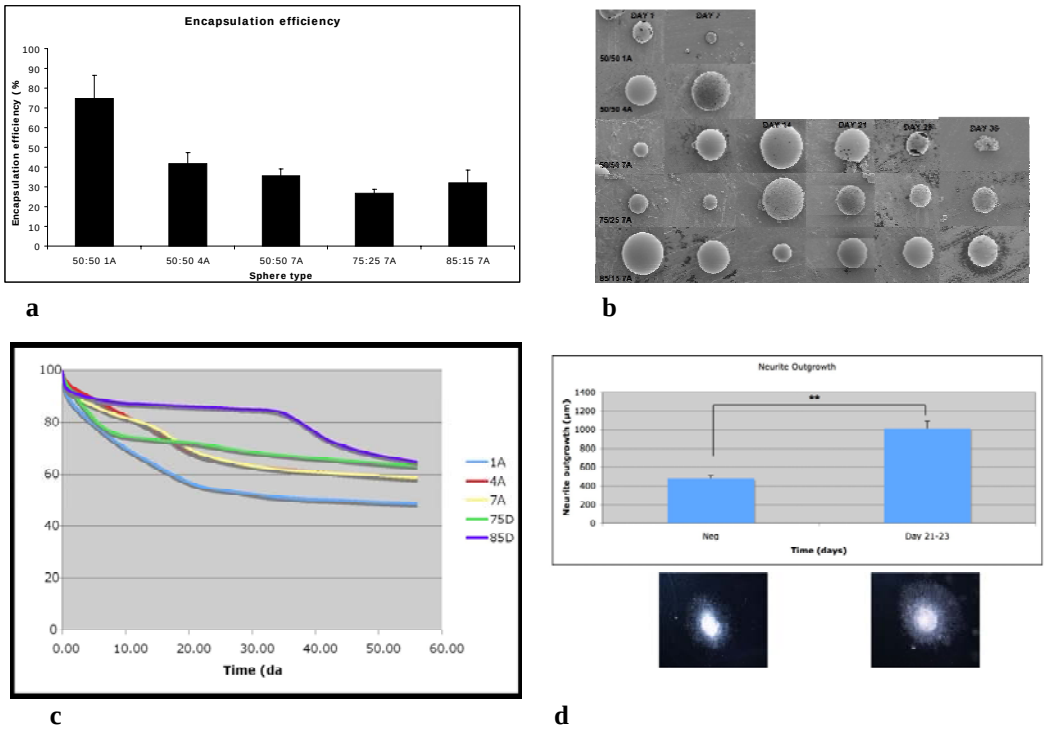


Fig. 2. *In vitro* release of NGF encapsulated in PLGA microspheres. (a) the encapsulation efficiency of the NGF ranged from 70% to 30% depending on the PLGA formulation. (b) Microsphere degradation was observed by scanning electron microscopy. (c) Controlled NGF release was observed over a period of 60 days for all PLGA compositions. Release data from figure 1c was obtained by ELISA, which used an antibody to 'active' NGF. (d) Activity of NGF was confirmed in dorsal root ganglion explant cultures grown on collagen coated plastic for 24 hours, with NGF microspheres in a 'transwell TM' (filter membrane 0.2µm insert). A significant increase in neurite outgrowth was observed emerging from the primary DRG explants in contact with media that contains NGF 50:50 microspheres for up to 3 weeks.

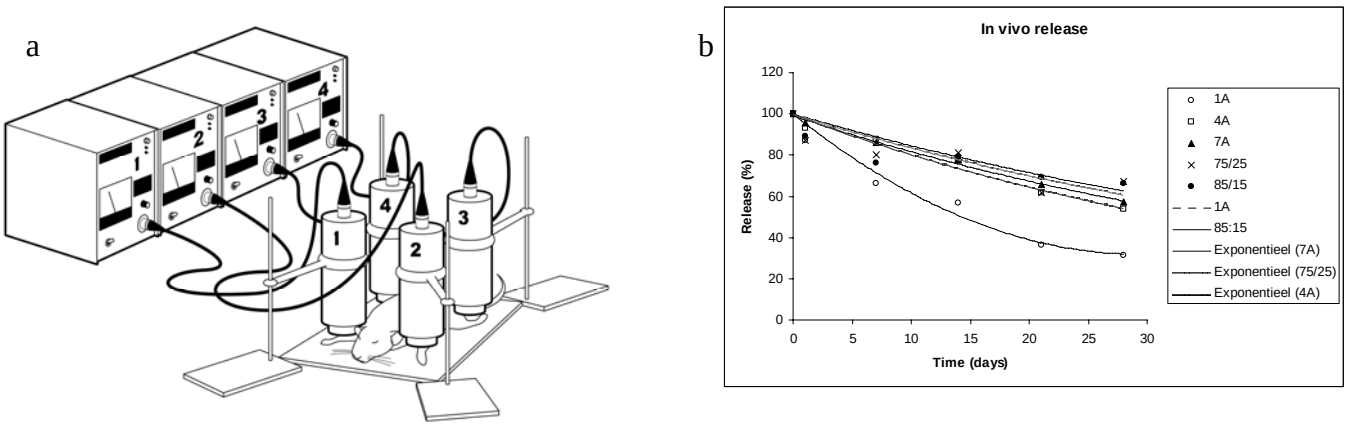


Fig. 3. 125I-NGF was encapsulated in PLGA microspheres. A microsphere slurry was placed within PLGA 85:15 hollow tube scaffolds with an internal diameter of 1.6mm and a length of 1.2cm. Scaffolds were placed between muscles in each limb of a 250g SD female rat. (a) shows the radioactive counts associated with the encapsulated 125I-NGF measured in each quarter of the rat. Based on the *in vitro* and *in vivo* release data (b), 50:50 PLGA microspheres encapsulating either NGF or GDNF were selected for the transected sciatic nerve regeneration experiments.

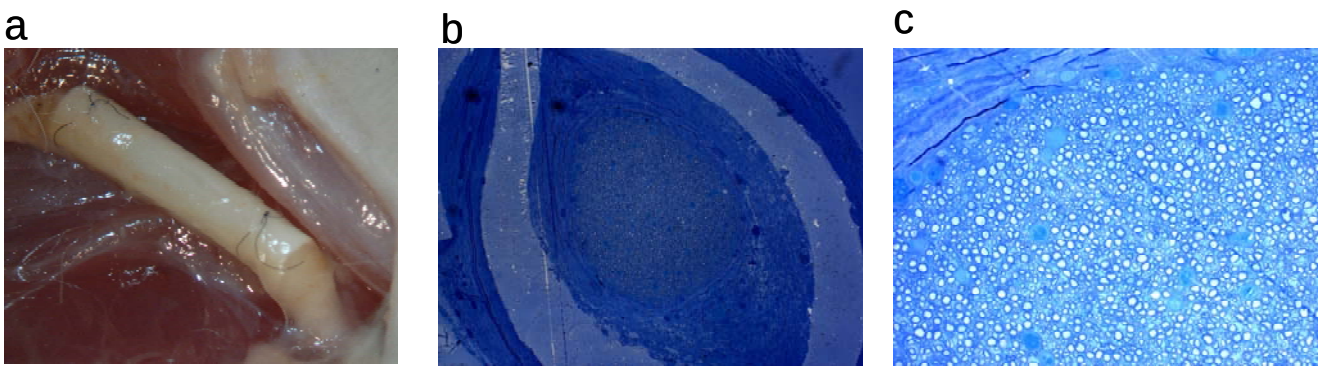


Fig. 4. Nerve tube after 17 weeks bridging a 10mm gap in the sciatic nerve
(a) PCLF tube in rat sciatic nerve. The tube has a 1.6mm internal diameter and 12mm long with 100µm wall thickness. (b) Neuronal regeneration in the sciatic nerve hollow scaffold, shown as a mid transverse section (5x objective). (c) The same section showing nerve regeneration with early remyelination (100x objective)

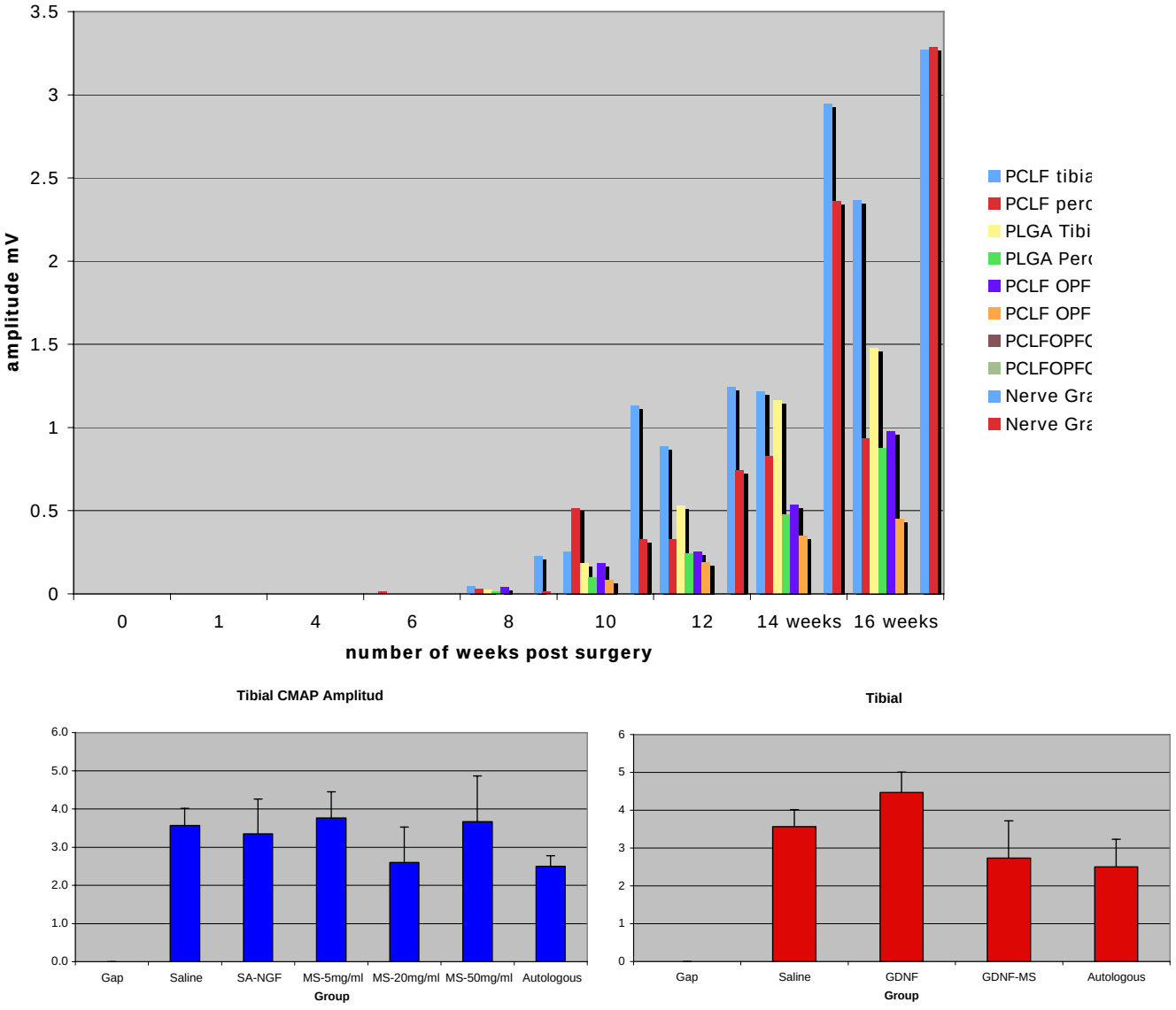
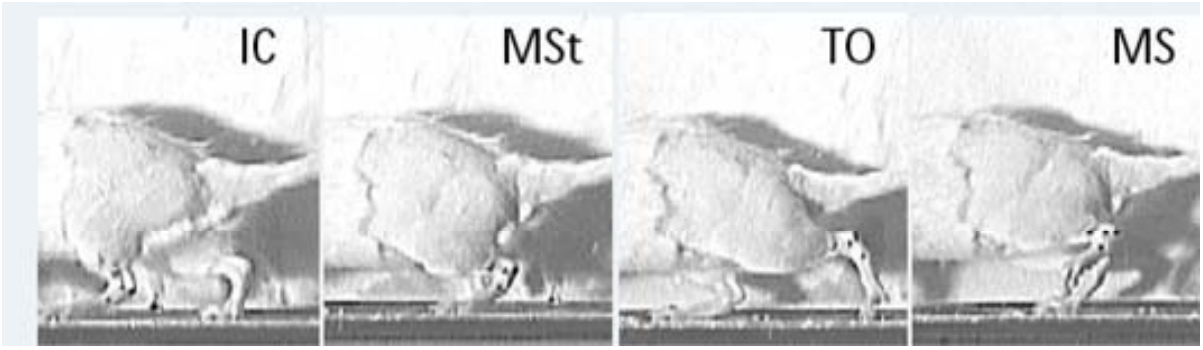
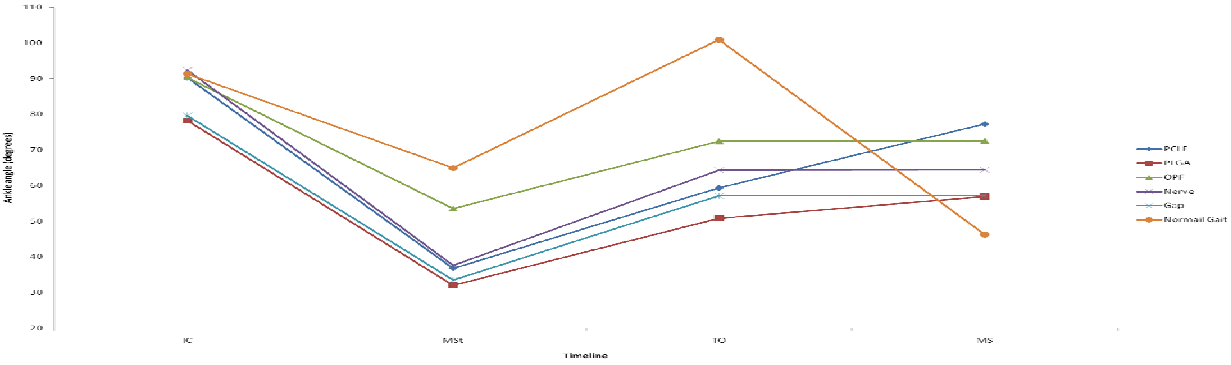


Fig. 5. Electrophysology after insertion of the sciatic nerve tube. (a) Electrophysiology after inserting scaffolds made from different polymers; PCLF, PLGA, PCLF-OPF or a nerve autograft to bridge a 10mm gap in the rat sciatic nerve. A negative control with an unrepaired nerve gap had no response after 16 weeks. The positive control (uncut nerve on the opposite leg) showed CMAP amplitudes 2-3 fold higher than the autograft at 16 weeks. (b) After 16 weeks hollow PLGA nerve tubes filled with NGF microspheres showed similar tibial CMAP data to the autologus autograft. (c) All were significantly higher than zero amplitude reading obtained by the nerve gap without a tube. The GDNF group produced the highest CMAP amplitude.





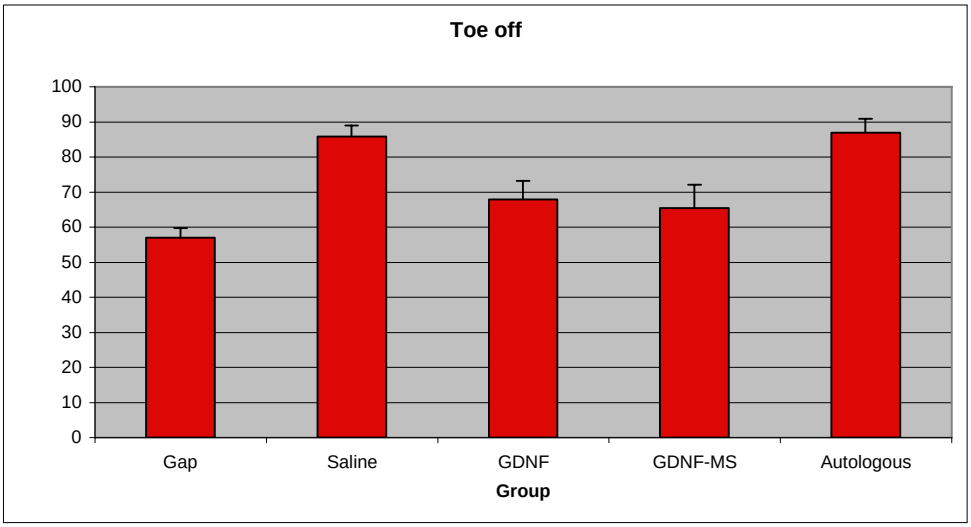


Fig. 6. Three different nerve tubes, autograft and nerve gap were compared with baseline (pre-surgery) over the 16 week observation time. For each animal four full walking cycles were observed. Figure (a) shows the four stages of the walking cycle. Figure (b) compares all four stages in the walking cycle at 16 weeks for the nerve gap group, autologous autograft and three polymer groups. (c) Statistical comparison of the ankle angle at 16 weeks for all groups at toe off. None of the nerve tubes or the nerve graft are returned to normal in terms of gait analyses. Together with the electrophysiology this would indicate that the observation period could benefit from extending in the future. Contractures may also influence these data. However, PCLF, OPF and the autograft showed better functional recovery than the nerve gap and PLGA.